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Refilin holds the cap

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The Refilins (RefilinA and RefilinB) are a novel family of short-lived actin regulatory proteins that are expressed during changes in cellular phenotype such as epithelial to mesenchymal transition (EMT). The Refilins promote the formation of actin- and myosin-rich perinuclear bundles that are characteristic of cellular phenotypic switches. In epithelial cells, RefilinB is upregulated in response to TGFβ stimulation and functions in organization of apical perinuclear actin fibers during early stage of the EMT process.[1] In fibroblasts, RefilinB stabilizes perinuclear parallel actin bundles which resemble actin cap.[2] Refilins bind and modulate the function of Filamin A (FLNA). Upon binding to Refilins, FLNA is capable of assembling actin filaments into parallel bundles, possibly by undergoing conformational changes at the C-terminal. Perinuclear actin structures determine nuclear shape, cell morphology, cell adhesion and possibly cell proliferation and gene regulation. Identifying the role of Refilins in organizing perinuclear actin networks provides additional insight in the process of intracellular mechanotransduction that regulate changes in cellular phenotype such as those observed during EMT.

Key words: FAM101A, FAM101B, RefilinA, RefilinB, cfm, Filamin, FLNA, actin cap, TAN line, LINC complex

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Refilin Proteins

The actin cytoskeleton is crucial for development as it controls cell division, membrane remodelling, cell migration and differentiation. These essential functions rely on the dynamic nature of the actin cytoskeleton. The mechanisms that determine actin cytoskeleton organization and dynamics are controlled by a wide array of regulatory proteins. In this context, we have identified a new family of short-lived actin regulatory proteins, the Refilins, which are expressed during cell differentiation switches and serve as organizers of perinuclear actin networks. There are two known Refilin isoforms, RefilinA and RefilinB. RefilinA has a half-life of less than one hour and is transiently upregulated during differentiation of rat neural multipotent precursor cells into glial progenitors (unpublished data), while RefilinB is expressed during epithelial-mesenchymal transition (EMT) mediated by TGFβ.[1]

Rat RefilinA and RefilinB proteins display 40% identity and 48% similarity (**Fig. 1**). Refilins are capable of forming homodimers; the dimerization domain is located at the monomer's N-terminus.[1] The stability of Refilins is determined by their N-terminal sequences; mutation of the N-terminal domain alters the half-life of Refilins (see **Fig. 1**, unpublished data). Refilins exhibit greater stability in cells treated with the protease inhibitor MG132, suggesting that Refilins are subject to proteasomal degradation (unpublished data). Given the obvious impact of Refilins on actin dynamics, the mechanisms that determine the stability and degradation of Refilins deserve further investigation.

Refilin Promotes FLNA-Dependent Actin Bundles

In cells, Refilins are stabilized upon interaction with filamins, which are actin-binding and cross-linking proteins. Vertebrate filamins are the only

Figure 1. Sequence alignment of rat RefilinA and RefilinB proteins. The two proteins show conserved regions with homologous (purple) or similar (blue) sequences. A 15 amino-acid N-terminal sequence is fully conserved between the two proteins, whereas a specific sequence is only found in RefilinB (red rectangle). These two regions function to control Refilin stability and degradation (manuscript in preparation).

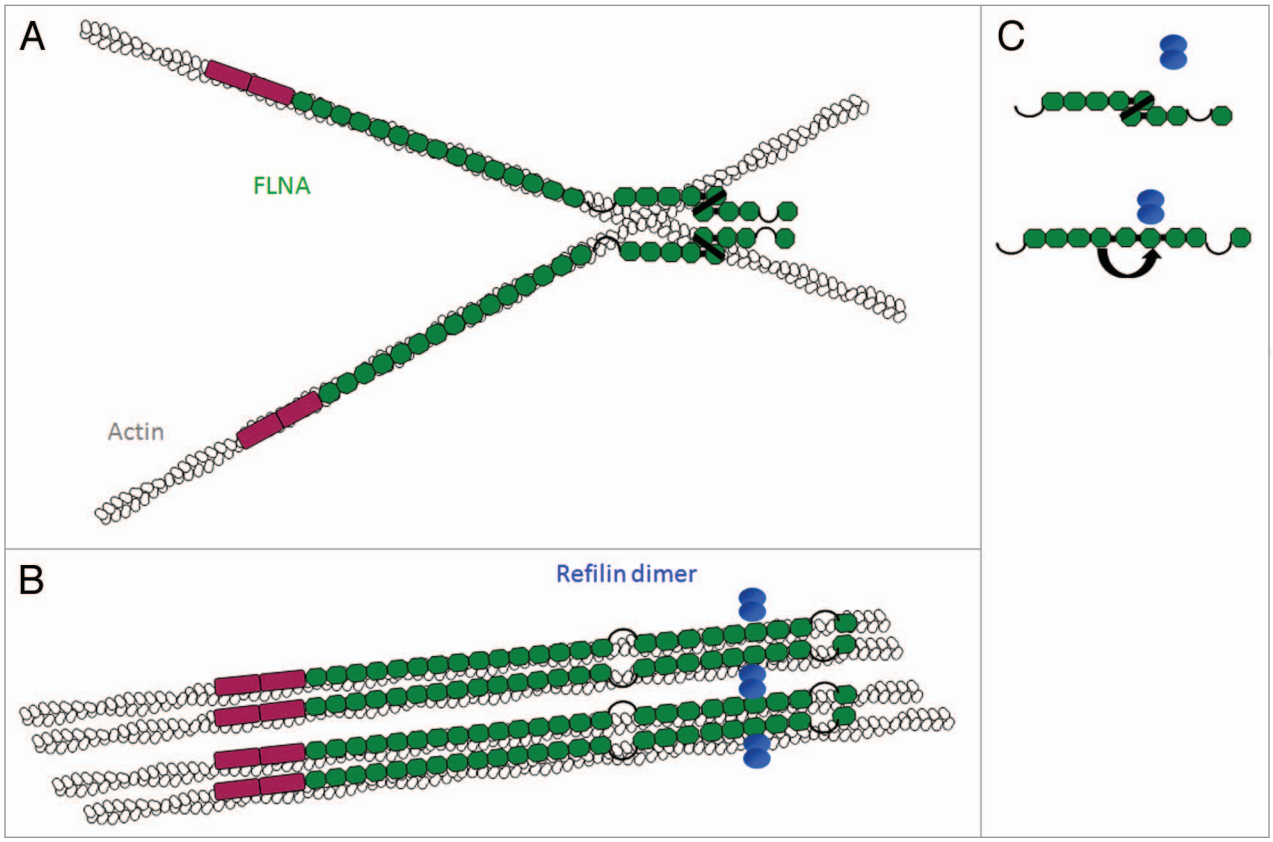

Figure 2. Refilin/FLNA complex organizes actin network into bundles. A proposed model by which Refilin binding promotes conformational changes in FLNA molecules that favor the bundling of actin filaments. (A) In the absence of bound Refilin, the V-shape FLNA molecule (green) generates actin networks (gray). (B) After binding of Refilin dimer (blue), FLNA acquires actin-bundling instead of actin-networking properties. (C) Domains 19, 20 and 21 on FLNA are folded such that domain 21 is entrapped between domains 19 and 20. The binding of dimeric Refilin to domain 21 of FLNA induces a conformational change of the 3 domains.

proteins known to co-immunoprecipitate with Refilins. Filamin A (FLNA) is the most abundant and best-characterized member of the three filamin isoforms. FLNA is ubiquitously expressed and provides mechanical stability to the actin cytoskeleton. FLNA also functions as a scaffolding protein for various cellular signaling pathways.[3] Vertebrate FLNA is a homodimer of 280 kDa subunits composed of an N-terminal actin-binding domain followed by 24 immunoglobulin-like domains.[4] Two intervening calpain-sensitive "hinges" separate the repeats into rod 1 (repeats 1–15), rod 2 (repeats 16–23) and the dimerization domain (repeat 24). A secondary F-actin-binding domain resides in rod 1,[4] whereas rod 2 does not interact with F-actin, leaving it free to associate with partner proteins (**Fig. 2**). In the absence of bound Refilin, FLNA dimers crosslink actin filaments into orthogonal networks.[4-7] The binding of Refilins converts FLNA from an actin branching protein into one capable of assembling actin filaments into parallel bundles (**Fig. 2A and B**).

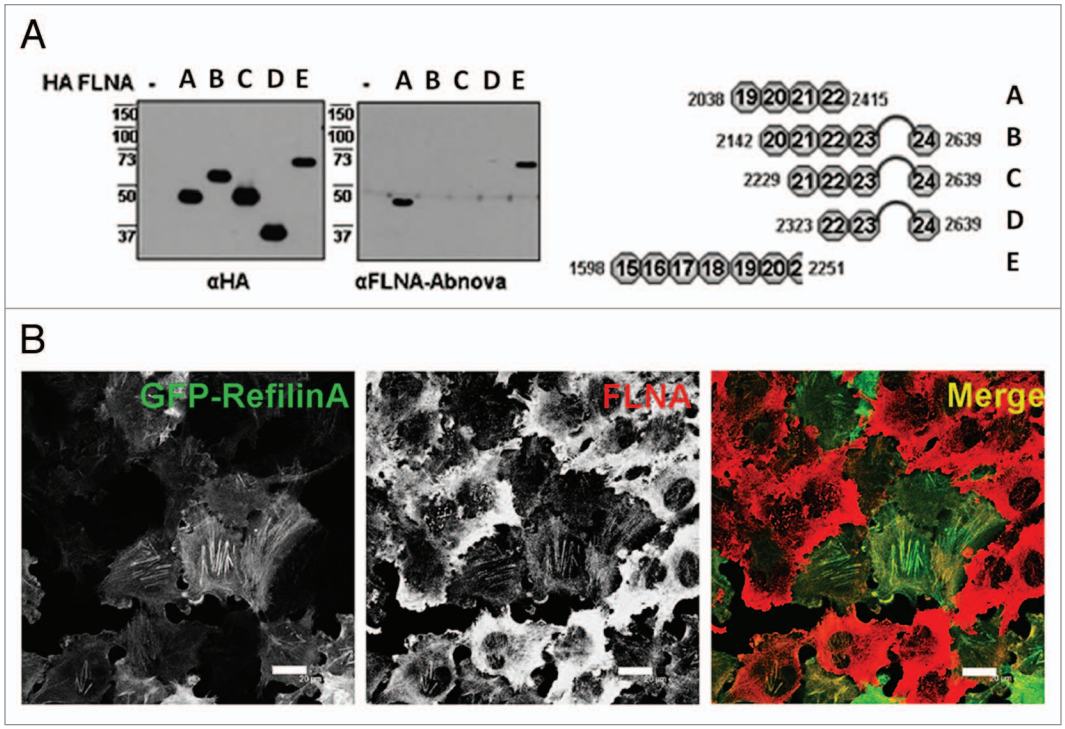

Figure 3. Monoclonal anti-FLNA antibody as a sensor of RefilinA-FLNA interaction. (A): Map depicting the epitopes of mouse monoclonal anti-FLNA. HA-tagged FLNA fragments (A–E) as indicated were expressed in human melanoma M2 cells and total cell lysates were probed with mouse anti-HA or anti-FLNA from Abnova. (B) U373 cells transfected with GFP-RefilinA plasmid were fixed and immunostained with an anti-FLNA antibody. Cells expressing GFP-RefilinA showed a drastic decrease in FLNA immunoreactivity. Bar = 20 μm.

Refilins enclose two putative FLNA binding domains and are capable of homodimerization at their N-termini.[1] It is therefore conceivable that Refilins crosslink FLNA and that crosslinked FLNA induces bundling. In this sense, RefilinA may function as a "zipper" that promotes the formation of multimolecular FLNA complexes on F-actin (**Fig. 2B**).

Moreover, three-dimensional structural studies revealed that the 19–21-domain fragment of human FLNA may function as an auto-inhibitory domain.[8,9] Based on detailed FLNA mutagenesis studies, we have proposed that Refilins bound to FLNA repeat 21 induce a conformational change in FLNA repeats 19–21 and change the high-angle F-actin branching mediated by FLNA into a low angle bundling of F-actin (**Fig. 2**). The notion that Refilin induces a conformational change in FLNA 19–21 domain is supported by the drastic decrease in FLNA immunoreactivity in the presence of RefilinA; this was determined by immunofluorescence using an antibody directed against repeat 19 of FLNA (**Fig. 3**).

Refilin/FLNA Complex Localizes on Actomyosin Fibers

Refilin binding to FLNA promotes actin bundling in vitro.[1] In U373 astrocytoma cells, overexpression of a GFP-Refilin fusion protein promotes FLNA translocation from actin fibers, membrane ruffles and the cytoplasm to well-defined basal actin stress fibers that are connected to focal adhesion sites (**Fig. 4**). In addition, the Refilin/FLNA complex also appears on parallel perinuclear actomyosin fibers that organize above the nuclei and control nuclear height (**Fig. 4B** x-z and reviewed in ref. 1). In NIH 3T3 fibroblasts, endogenous RefilinB is expressed in confluent cells and the protein co-localizes with FLNA on perinuclear actomyosin fibers.[1] Co-localization of RefilinB with FLNA is also observed in basal stress fibers in over-confluent NIH 3T3 cells (unpublished data). In mouse epithelial NMuMG cells, RefilinB is expressed in response to TGFβ stimulation and the RefilinB/FLNA complex is specifically located on perinuclear actin filament bundles that form at the apical surface, but not on basal actin fibers. Collectively, these observations suggest that in the presence of low concentrations of RefilinB, the RefilinB/FLNA complex shows more avidity for the apical perinuclear actin cytoskeleton, although the RefilinB/FLNA protein complex may also localize to conventional basal actin fibers.

What are Perinuclear Actin Structures?

Two perinuclear actin structures have recently been described: the "actin cap"[2,10] and the transmembrane actin-associated nuclear (TAN) line.[11] Actin caps generate tension in order to control nuclear shape while TAN lines are involved in nuclear positioning during cell polarization.

Both the actin cap and the TAN line are positive for myosin staining although they exhibit two main differences. First, the actin cap and TAN lines are oriented parallel and perpendicularly with the direction of cell migration, respectively. Second, actin caps are directly linked to focal adhesions; this is not observed in TAN lines. It is important to note that actin caps have been identified

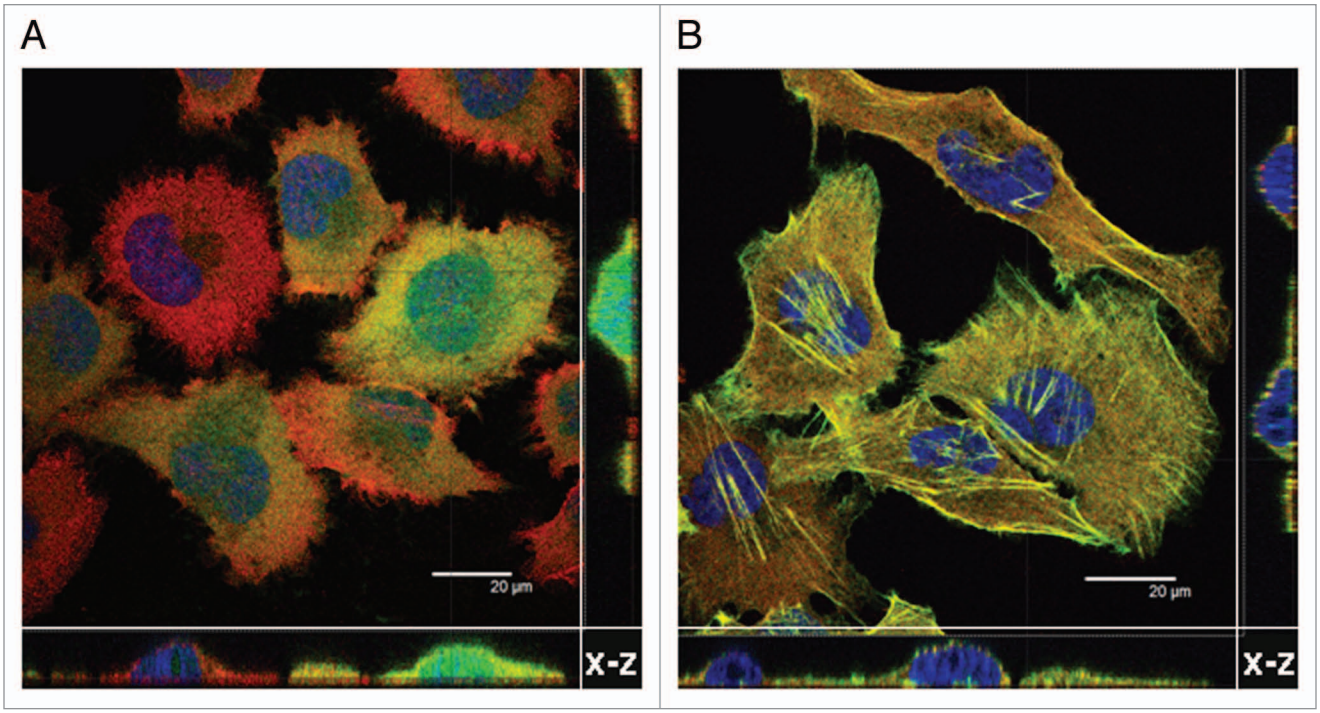

Figure 4. Localization of the Refilin/FLNA complex in U373 MG cells. U373MG cells infected with recombinant adenovirus expressing GFP (A) or recombinant RefilinB-GFP (B) were fixed in paraformaldehyde and immunostained with antibodies against FLNA from USBiological (red). RefilinB forms a complex that can be found on basal stress fibers but also on apical perinuclear actin structures (x-z). Bar = 20 µm.

in cells plated on micropatterned substrata[2] whereas TAN lines were observed in NIH3T3 fibroblasts migrating into scratch wounds.[11] Despite these differences, both actin caps and TAN lines-associated actin are linked to the nucleus by a specific set of proteins termed the LINC complex (linker of nucleoskeleton and cytoskeleton).[12]

In our studies with U373 and NIH 3T3 cells, the perinuclear actin structures associated with the Refilin/FLNA complex present characteristics of the actin cap. In epithelial NMuMG cells stimulated by TGFβ, the RefilinB/FLNA complex also contributes to the organization of apical perinuclear actin that accompanies the early stages of EMT. The identification of this novel perinuclear actin network provides additional insight into the mechanisms that regulate changes in cellular phenotype such as those observed during EMT.

Conclusions

In eukaryotic cells, the actin perinuclear structures control nuclear movements and cell adhesion, which are essential functions for development. These structures may also influence gene expression and their presence correlates inversely with cellular proliferation.[13,14] The identification of an actin regulatory protein complex (Refilin/FLNA) that organizes perinuclear actin during changes in cellular phenotype has furthered our understanding of the role of perinuclear actin in normal and pathological situations. In **Figure 5**, we propose a model outlining the putative function of the Refilin/FLNA complex. The role of this protein complex in perinuclear actin organization requires further investigation.

Acknowledgments

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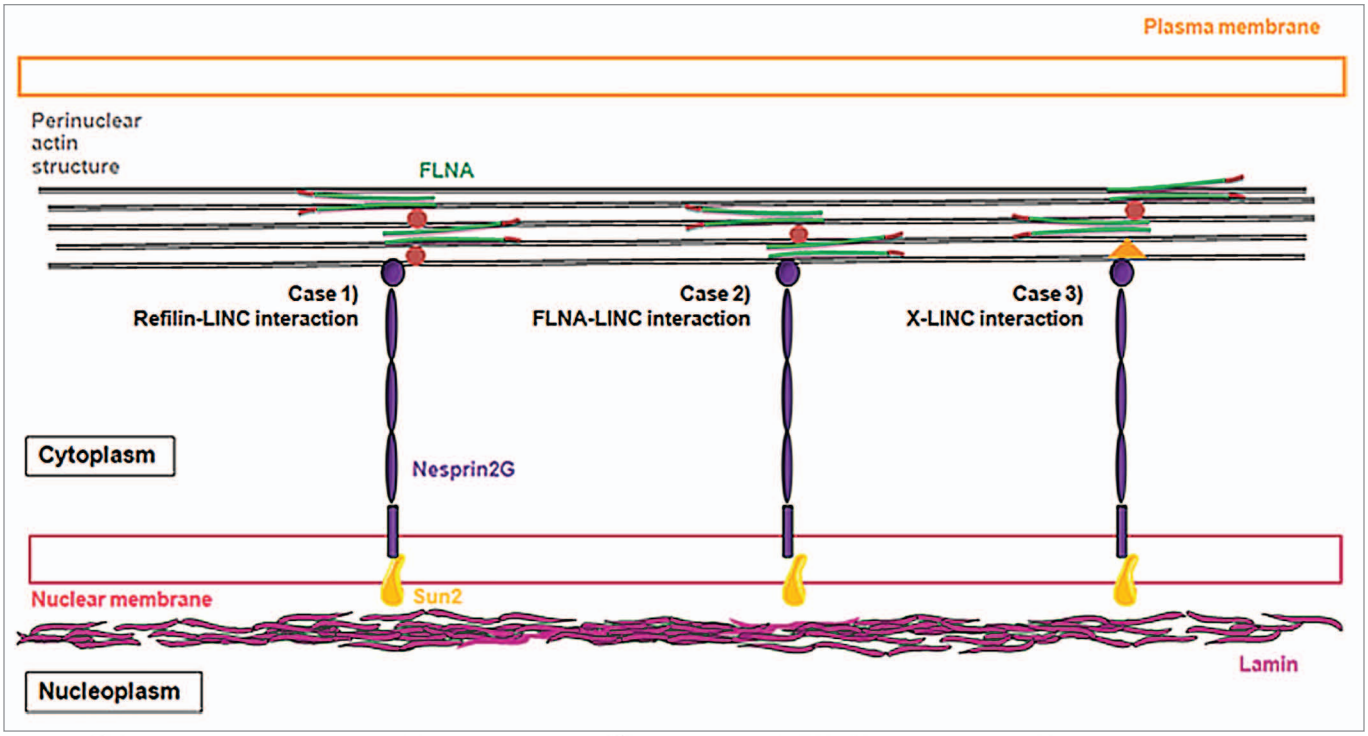

Figure 5. Hypothetical models of actin perinuclear structures stabilization by Refilin/FLNA complex. Schematic representation of the hypothesized roles of Refilin and FLNA in the formation of perinuclear actin structures. After binding to Refilin, FLNA changes from an actin-crosslinker to an actin bundler and the Refilin/FLNA complex subsequently organizes perinuclear actin structures by interacting with components of the LINC complex through binding to Refilin (blue), FLNA (green) or a presently unidentified protein (X, orange). These hypotheses will require further investigation.

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